

# SYMPOSIUM ON MULTIPLE FORMS OF ENZYMES AND CONTROL MECHANISMS<sup>1</sup>

## III. CONTROL BY REPRESSION OF A BIOCHEMICAL PATHWAY

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### INTRODUCTION

A biochemical pathway may be defined as the specific sequence of reactions leading either to the synthesis of a building block of the cellular macromolecules (for instance, arginine synthesis stemming from glutamic acid) or to the conversion of a metabolite into an intermediate of a main metabolic pathway (for instance, the insertion of galactose metabolism into the glycolytic scheme). Most of these sequences have been elucidated without any feeling that the lining up of the corresponding enzymes in a path with directional arrows was more than a device to indicate the chemical relationship among members of the same reaction sequence.

The first indication that the group of enzymes participating in the synthesis of a given metabolite may indeed correspond more closely to a biological unit came from studies devoted to locating along the chromosome the genetic elements responsible for enzyme structure. The observation, made by Demerec and Hartman (5) some 6 years ago, was that the genes responsible for the structure of enzymes belonging to a given chemical pathway were, at least in some instances, clustered in a small region of the chromosome, lined up in the same sequential order as the corresponding chemical reactions, and probably adjacent to each other. This discovery came to most as a surprise [not to Pontecorvo who, since 1950, considered the possibility of "an orderly arrangement of genes acting in series upon suc-

cessive steps of a chain of biochemical reactions" (22)]. Actually, it gave the first hint that the arrows on the blackboard may define a real biological entity, at least at the level where genetic information is secured.

A few years later, studies (29, 12, 1) of the mechanism by which a cell controls the rate of synthesis of its own enzymes demonstrated that this control may be divided into units, each one specifically governing the rate of synthesis of a group of enzymes. These groups are delimited by the biochemical pathway to which the enzymes specifically belong. The units of a biochemical pathway may thus be defined in two ways: as genetic and as control linkages. I shall start by analyzing the linkage defined by the control mechanism.

### BIOLOGICAL UNITS DEFINED BY THE CONTROL MECHANISM OF BIOCHEMICAL PATHWAYS

Taking the arginine biosynthetic pathway as an example, it has been found (27, 10, 16) that the synthesis of each enzyme of the pathway is specifically and independently repressed by arginine, the end product of the pathway. Repression is specific in the sense that none of the intermediates exert this repressive effect, and independent in the sense that a genetic block interrupting the pathway at any point does not prevent the end product from repressing the enzyme or enzymes before the block. It was also established that the specificity of repression is reciprocal: the arginine-biosynthetic enzymes are repressed only by arginine, and arginine represses only the arginine-biosynthetic enzymes. Thus, the biochemical pathway of arginine indeed defines a biological unit at the level of the synthesis of arginine enzymes.

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This unit is a unit of control, arginine being the common repressor of the biosynthetic activity of the entire unit. The same is true for the biosynthetic units of every building block (amino acid, purine, or pyrimidine) which has been tested so far: each is controlled through repression by its own end product. The existence of such units of control is not confined to repressible systems but is characteristic of inducible pathways as well. For example, the enzymatic pathways which convert lactose or galactose into Embden-Meyerhof intermediates are specifically and independently induced as a unit by lactose (20) or galactose (28), respectively. Generally speaking, one can affirm today that all the control systems which have been studied are units composed of differing numbers of enzymes, depending upon the length of the specific metabolic chains. The synthesis of the individual enzymes of the unit is controlled simultaneously, rather than sequentially, by the same repressor or inducer.

This symposium deals with a very interesting aspect of this compartmentalization of control mechanisms: the existence of multiple forms of enzymes.

As one of the preceding speakers has clearly shown, when different pathways share a common intermediate in the same cell, one may find multiple enzymes that catalyze the same reaction (25, 23). These are the same enzymes as defined by chemical activity; they differ, however, because the synthesis and activity of each is regulated in a different way, i.e., by different control systems. Thus, as has already been pointed out (3), it is more valuable for the survival of a strain to develop separate control units without mutual interference than to spare the synthesis of an enzyme which, from an old-fashioned, merely chemical point of view, is a duplicate. It appears, therefore, that for the sake of coordination among different control units a cell may produce as many proteins with the same enzymatic activity as are needed to satisfy each pathway possessing the same intermediate.

I should add that when, on the contrary, one does find an interference between two pathways, the existence of a coordination with a purpose different from building-block synthesis should be seriously considered. For instance, it is possible to demonstrate in *Escherichia coli* an interference in arginine synthesis by uracil. Addition of uracil to a culture of a wild-type strain growing in mini-

mal medium causes a reduction in arginine synthesis. This is indicated by a partial release in the presence of uracil of repression of the arginine biosynthetic enzymes in an arginine-repressible strain. The level of ornithine transcarbamylase (the enzyme converting ornithine to citrulline), for instance, increases two to three times (11). This indicates that the size of the arginine pool has been reduced under these conditions. We have evidence that this effect of uracil is exerted by repression of the enzyme(s) involved in the biosynthesis of carbamyl phosphate, a common intermediate for both the arginine and the uridylate pathways. The observed interference suggests, perhaps unexpectedly, that only one form of enzyme may be shared by these two pathways. The rationale of such interference between arginine and uridylate synthesis could be that it provides a mechanism for coordination of ribonucleic acid (RNA) and protein synthesis. It may be that such coordination is effected by interference in the synthesis of an amino acid by a pyrimidine, at the level of a common precursor, since it is known (21, 13) that the absence of only one component of the amino acid pool is sufficient to stop not only protein but also RNA synthesis.

To conclude this consideration of control units as biological entities, I would like also to add a remark of heuristic value. As Umbarger (24) pointed out, the existence of a control unit offers an easy approach for solving certain problems of intermediate metabolism. One can infer whether an enzyme catalyzes a step in a given pathway not only by a laborious tracer analysis but more simply by ascertaining whether this enzyme obeys the control system specific for the pathway.

In this paper, I would like to discuss some of the problems posed by the existence of these control units in connection with the results obtained in our laboratory by studying control mechanisms in the arginine-biosynthetic pathway. These problems are (i) the recognition by the repressor of its site of action, (ii) the relation between repressor and inducer in a single system, and (iii) the superposition of different repression mechanisms in the same system.

#### RECOGNITION BY THE REPRESSOR OF ITS SITE OF ACTION

One must distinguish from the beginning between substances which repress enzyme formation when present in the medium, and the hypothetical

macromolecules, which we may designate the "holorepressors," that are active at the molecular level in the control of enzyme synthesis. The latter are undoubtedly complex and presumably include the former, or some metabolic derivative of them, in their structure.

In cases of both inducible and repressible enzymes, mutants have been isolated in which enzyme synthesis occurs at the maximal rate regardless of the presence or absence in the medium of normally inducing or repressing substances. Such mutations from inducibility or repressibility to constitutivity of synthesis might occur by one of two mechanisms: the holorepressor molecule might fail to be synthesized, or be synthesized in altered form; the site of action of the holorepressor might be altered in such a way as to change its affinity for the holorepressor. Mutants of the first type are called R or repressor mutants, of the second type O or operator mutants.

In a thoroughly studied case, that of  $\beta$ -galactosidase (14), constitutive mutants have been isolated of both the O and the R types. These types may be distinguished operationally, on the assumption that the site of repressor action is the gene itself, by the *cis-trans* test: in a merodiploid or merozygote, the O mutation should be dominant, the R mutation recessive, vis-à-vis the wild-type, inducible allele. The genetic sites responsible, respectively, for the synthesis of the holorepressor molecule and for its action have been identified and mapped. They have been found to be in distinct but adjacent regions of the *E. coli* chromosome, near to but genetically separable from the gene which determines the structure of the  $\beta$ -galactosidase molecule. Almost nothing is known as yet about the structure of the repressor molecule in the  $\beta$ -galactosidase system.

In the arginine system of *E. coli*, a genetic element responsible for the mutation of the entire arginine pathway from repressibility to constitutivity has been located far from any one of the structural genes for the known arginine enzymes (10, 16). It has not yet been possible in this system to apply the *cis-trans* test to distinguish whether the observed mutations are of the R or the O type. However, as will be seen, the scattering of the arginine structural genes renders the first alternative far more likely, since the second would require that a single mutation cause a change in the structure of many sites along the chromosome, something unprecedented in bacterial genetics.

The number of operators per control unit is at present a controversial point. To account for the simultaneous repression of all the enzymes of a unit, there might be either a single operator per control unit or as many identical copies of it as there are enzymes in the unit. A control by only one operator should have a better survival value than one by several operators, because the cell has to manufacture only one molecule of repressor per unit time instead of several. This is mere teleology, but some operational inferences can be drawn from either possibility. If only one operator per sequence exists, mutants with altered repression for only one enzyme of the sequence are theoretically impossible. As a matter of fact, such a mutant has not been found as yet in any control unit. However, it should be pointed out that as yet no effective method for selecting mutants with altered control in only some of the enzymes of a sequence has been developed.

A second, more interesting implication is the following. If the synthesis of all the enzymes of a sequence is controlled through a single operator, at one point in the synthetic process a single reaction must be involved for all the enzymes of the sequence. In other words, to avoid the necessity of invoking a mysterious mechanism of action at a distance, it seems to me that a single operator requires the simultaneous transmission of information for the whole sequence if not a single template per sequence. We may again recall in this connection the discovery of Demerec (4) that frequently the genes corresponding to a given pathway are located in clusters. In part based on this finding, the well-known hypothesis of Jacob and Monod (14) postulates that the single step per sequence is the formation of messenger RNA and that the repressor acts at this step through a single site of action or operator.

According to this hypothesis, the chromosome is divided into sections, each one synthesizing as a unit the messenger RNA corresponding to the entire set of enzymes of a biochemical pathway. Each chromosomal unit is called an operon and contains its specific operator. Thus, according to this hypothesis, the real unit of control which I have defined only operationally at the beginning of this paper would be a segment of chromosomal deoxyribonucleic acid corresponding to the definition of the operon.

To have general validity, this hypothesis requires that all the control units should have their structural genes located in a cluster and adjacent

to each other. In fact, this genetic contiguity is not a general situation. To speak only of the few control systems which have been studied, the arginine (10, 16) and the uridyate (2) pathways do not obey this requirement; the eight structural genes of the eight arginine enzymes, for instance, are scattered in at least five places along the chromosome.

One may therefore conclude that the operon model does not account for all the control systems. It is quite possible that one cannot answer the question as to how a repressor recognizes its site of action by postulating only one pattern. An indication that two mechanisms may exist, one controlled through an operon and another through several copies of a repressor-sensitive site, is furnished by the study of the quantitative extent of repression reached by the different enzymes of a sequence. It appears that in the sequences for which an operon can be postulated, for instance, the histidine (1) or galactose (28) genetic clusters, all the enzymes are controlled coordinately; i.e., the levels of the different enzymes of these sequences vary proportionately under various conditions of repression or induction. One should expect such a coordination if the control is exerted through a single operator. By contrast, in the systems for which an operon cannot be postulated, for instance, the arginine or uridyate system in which the structural genes are totally or partially scattered, a coordinate repression is not observed, several enzymes of the sequence being repressed to different extents under various conditions (27, 10, 16, 2). However, notwithstanding this lack of quantitative coordination, it remains to be made clear how the arginine or the uridyate control system can act as a unit in spite of its loose topographical ties.

#### RELATIONSHIP BETWEEN REPRESSION AND INDUCTION

The history of control mechanisms began with the study of enzyme induction upon addition of an external inducer (19). Later, enzyme repression upon addition of external end products was discovered (26), and shortly thereafter it was recognized that repression is also exerted by endogenously produced end products (29, 12). The bridge connecting induction and repression became apparent in the inducible  $\beta$ -galactosidase system when Pardee, Jacob, and Monod (20) performed a now classical experiment which indicated that the wild-type, inducible strains differ

from constitutive mutants in producing an endogenous repressor of  $\beta$ -galactosidase synthesis rather than in lacking an internal inducer. The presence of this as yet unidentified repressor is the reason these strains require an external inducer. Thus, induction and repression appear to be two sides of the same coin. In no single case, however, has it yet been possible to look at the coin from both sides at once. In the system with a known inducer, the repressor is only postulated, and an inducer has never been demonstrated in a repressible system. The situation is somewhat like that in atomic physics in which you may know precisely either the speed or the position of a particle, but never both at the same time.

Presumably, this failure in the study of induction and repression is not as insurmountable as the Heisenberg uncertainty principle in atomic physics. At least this was our presumption when we made use of a control unit with a known repressor to test some specific points (8; Gorini and Wilson, *unpublished data*). Assuming that repression is the primary phenomenon and induction is only a counteraction of repression, it is evident that any structural analogue of a repressor, which lacks repressing activity, might behave as an inducer by competing with the repressor. This model of induction disposes of the inducer as an independent biological entity and renders its function dependent upon its chemical resemblance to the repressor. One may question, however, whether this is the only way in which induction occurs, and it would seem wise to look for substances which act as inducers because of some specific metabolic relationship with the control unit they induce rather than because of their structural analogy with the repressor.

The arginine pathway may be considered as an

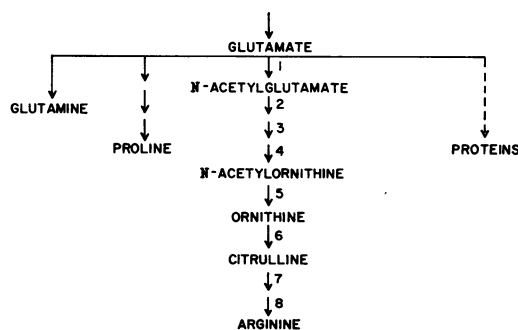


FIG. 1. Role of glutamate in the production of arginine and other precursors of proteins.

example (Fig. 1). The specific biosynthesis of arginine starts from glutamate. Besides being a precursor of arginine, glutamate itself serves directly as a building block for proteins and as a precursor of glutamine and proline. Arginine is synthesized from glutamate in eight steps. Several of the intermediates (acetylglutamate, acetylornithine, ornithine, and citrulline) are stable and readily obtainable, and enter into the cell without difficulty if permeable strains are employed. Using mutants with two blocks in the arginine pathway, one immediately before and one after the intermediate under consideration, one can test the inducing effect of each intermediate under conditions in which it is not formed or transformed endogenously. Repressible and constitutive (i.e., nonrepressible by arginine) strains were compared. The repressible strains were grown in the chemostat under steady-state limitation by arginine adjusted so as to obtain (i) a condition of partial repression and (ii) a condition of complete derepression. Figure 2 shows the degree of derepression of ornithine transcarbamylase (OTC), which converts ornithine to citrulline (Fig. 1, enzyme 6), obtained when ornithine or acetylglutamate is added to a steady-state culture which is partially repressed by arginine. When ornithine is used, the mutant is blocked in enzymes 5 and 7, and, in the case of acetylglutamate, in enzymes 1 and 2. It is evident that ornithine or citrulline, or both (because ornithine

and citrulline are interconverted by OTC), counteract repression of OTC by arginine, whereas acetylglutamate does not. Acetylornithine behaves like acetylglutamate. Two other enzymes of the arginine sequence, acetylornithinase (enzyme 5) and argininosuccinase (enzyme 8), have also been examined. It was found that both behave like OTC. Finally, it was also found that ornithine or citrulline did not affect the enzyme level of a constitutive strain or of a repressible strain under conditions of complete derepression. Apparently, ornithine or citrulline, or both, counteract the repression by arginine of the entire biosynthetic sequence, whereas acetylglutamate and acetylornithine do not. Since the structures of citrulline and ornithine are analogous to that of arginine, whereas those of acetylglutamate and acetylornithine clearly are not, it appears that a structural analogue of the repressor may specifically reverse repression. One concludes that repression is indeed the primary act and induction merely a reversal of it.

It would appear, however, that induction by ornithine (or citrulline) must be physiologically unimportant, since ornithine is an intermediate of the sequence and its synthesis depends on that release of repression which it is here supposed to actuate. If an inducer in the form of an independent biological entity is required to derepress the arginine pathway, we would more logically expect it to be glutamate, since glutamate is the substrate from which the specific arginine pathway originates.

Preliminary results of experiments which will be published at a later date (Gorini and Silver, *unpublished data*) are consistent with the hypothesis that glutamate is an inducer of the arginine pathway. Since glutamate is itself a building block for proteins, one cannot perform an experiment analogous to those described earlier to determine the inductive effect of this compound. However, one may study the effect of glutamate on derepression of the arginine pathway in resting cells in the absence of net protein synthesis but where new molecules may be synthesized by protein turnover. The mutant used is unable to form glutamate because it is blocked in the condensing enzyme of the trichloroacetic acid cycle, and it is unable to transform glutamate into any intermediate of arginine biosynthesis because it is blocked in the first enzyme of the arginine pathway, acetylglutamate synthetase. In addition,

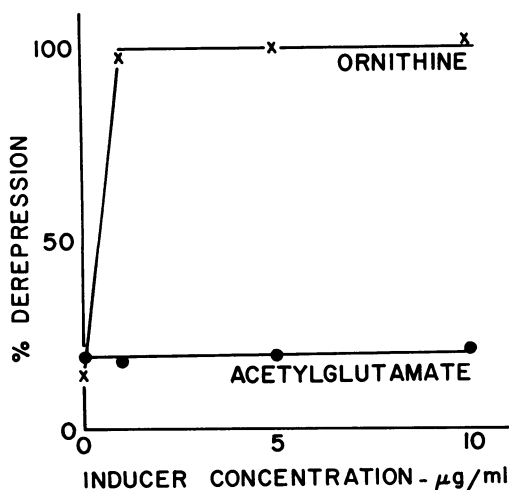


FIG. 2. Comparison of the effects of ornithine and acetylglutamate in derepressing ornithine transcarbamylase.

TABLE 1. *Arginine-glutamate competition in ornithine transcarbamylase derepression*

Arginine  <i>μg/ml</i>	Glutamate ( <i>μg/ml</i> )		
	0	10	100
0	2.2*	7.0	10.3
0.01	2.0	7.4	10.4
0.05	1.4	5.7	9.5
0.10	0.9	4.6	8.2
0.50	0.6	1.8	4.0
1.00	0.8	0.6	1.3

\* The figures represent specific activity of ornithine transcarbamylase (enzyme units/mg of protein). Time zero = 0.8.

the mutant is histidine-requiring, so that by omitting histidine from the incubation medium one can be assured that the amino acid pool of the bacteria will be deficient in at least one component irrespective of the presence or absence of arginine or glutamate. When a suspension of repressed cells of such a mutant in a medium without arginine and histidine is incubated at 37 C, derepression of arginine enzymes occurs only when glutamate is added to the medium. Two enzymes of the arginine pathway have been examined, OTC and argininosuccinase; both increase during the first 45 min of incubation, only when glutamate is present. In the absence of glutamate, this increase is not observed even after 20 hr. In control experiments, it was shown that the presence of glutamate has no effect on derepression of enzymes of histidine or uridylylate biosynthesis. Derepression of these enzymes always occurs to the same extent irrespective of the presence or absence of glutamate. Since the uridylylate and histidine pathways do not start from glutamate, this result is in accordance with our hypothesis regarding the nature of inducers of biosynthetic pathways.

As in the case of induction by ornithine, one can demonstrate that glutamate does not increase the level of OTC in a constitutive strain and that glutamate induction is competitive with arginine repression (Table 1).

Although these results are very promising, we do not consider them to be definitive as yet. If, however, our findings are satisfactorily confirmed, they will furnish a model for the regulation of a control unit by the opposed influences of repression (by the end product) and derepression (by the first substrate). The use of the term "de-

repression" rather than "induction" is deliberate, since in the absence of repression the unit appears to be able to function at its full potential.

Given the fact of the structural dissimilarity between the first substrate and the end product in many control units, especially when these compounds are several enzymatic steps apart, as in the case of the arginine system, it appears most unlikely that these molecules compete at a single binding site. A model which accounts for competition between two molecules without involving a common binding site has recently been proposed in connection with the phenomenon of feedback inhibition. It has frequently been found that the catalytic activity of the first enzyme of a specific biosynthetic sequence is competitively inhibited by the end product of the sequence, even in cases where there is a total lack of chemical resemblance between the first substrate and the end product. It has been suggested (7) that such competition between structurally unrelated molecules may be attributed to a distortion of the enzyme molecule produced by binding of the inhibitor at a site distinct from the catalytic site, resulting in a lessened affinity of the enzyme for the substrate. [Jacob and Monod (15) have recently proposed that the term "allosteric" be applied to proteins which participate in such phenomena.]\* Our own experimental results can be explained in a similar fashion if it is supposed that the aporepressor is a protein molecule whose affinity for the corepressor is diminished by binding of the inducer at a separate site.

#### DIFFERENT REPRESSION MECHANISMS FOR THE SAME PATHWAY

A feature peculiar to the arginine control system is the existence of the wild-type *E. coli* strain B in which the arginine-biosynthetic pathway is not repressible by arginine (8). Actually, the enzyme level of the entire arginine sequence in wild-type B is as low as in the repressible strains K12 or W. On the other hand, mutants can be derived in one step from strain B in which the level of the entire sequence is changed to the high value of a constitutive strain. It appears, therefore, that the synthesis of arginine enzymes in B is indeed under control, but this control is different from end-product repression. A better insight into this type of control was permitted by the observation that the level of the arginine enzymes in

\*Added in proof.

strain B is more dependent on the carbon source used for growth than it is in the repressible strains W or K12. In glucose, the enzyme level is the lowest (9). In wild-type strain B itself, this glucose effect is modest, but derivatives of strain B have been obtained in which the enzyme level may increase by a factor of ten or more when the cells are grown on glycerol instead of glucose. To conclude, strain B, although nonrepressible by arginine, is by no means a constitutive strain, judged by the criteria of both absolute value and variability of enzyme level. It is suggested that in strain B the synthesis of arginine enzymes is sensitive to repression by a product, "X," formed via general metabolism, especially from glucose. It is possible, however, that, in addition to this cytoplasmic factor, some other unknown genetic element is also responsible for the low enzyme level in wild-type strain B.

By genetic recombination of strain B, which is repressible by X, with strain K12, which is repressible by arginine, it has been possible to show that the arginine structural genes derived from K12 are under control by X when introduced into strain B and, conversely, that the arginine structural genes of strain B are under control by arginine when the R gene of K12 is introduced into strain B. In these recombination experiments, rare recombinants with either both controls or with neither of them were also obtained. There exist, therefore, two regulatory genes, R<sub>arg</sub> and R<sub>x</sub>, which are distinct from the structural genes and distinguishable from each other but very closely linked, and whose effects are additive in the same cell (10).

There is a striking similarity between the repression by X observed in the arginine biosynthetic pathway of *E. coli* B and the "glucose effect" [or "catabolite repression," as it has been more recently defined by Magasanik (18)] observed in several catabolic sequences leading to intermediates of the glycolytic pathway. Even more striking is the observation that this repression by X is counteracted by arginine. Thus one finds, astonishingly, that arginine may act formally as an inducer of its own biosynthetic pathway. This induction by arginine is modest (two- to threefold) in wild-type B, but is tenfold or more in those derivatives of strain B which display a glucose effect of ten times or more (9).

If one considers arginine solely as an end product of the induced sequence, it does not seem possible to explain this effect. I should say it is

"unfair" that an end product of a biosynthetic pathway should induce its own sequence and indeed this has not been observed in any other case. On the other hand, we have examples of amino acids which induce their own degradative pathway. For instance, the catabolic sequence degrading histidine in *Aerobacter aerogenes* (17) is induced by its corresponding amino acid; the induction is not sequential (the entire pathway is simultaneously induced) and appears to be a reversal of a catabolite repression. All these features are exactly reproduced in the induction by arginine observed in *E. coli* B of at least the last four enzymes of the biosynthetic pathway. If, for the sake of clarity, one supposes for a moment that arginine could be degraded in strain B by a reversal of its biosynthetic pathway, then arginine induction in this strain could fit with current ideas. Arginine would then bear the same relationship to the enzymes it induces as histidine bears to histidinase, urocanase, and the two other enzymes of its degradative pathway.

Of course, it is well known that neither *E. coli* B nor any other *E. coli* can use arginine as a source of carbon. Also, to my knowledge, no organism capable of degrading arginine uses a reversal of the biosynthetic pathway to do so. On the other hand, no logical reasons can be found to justify the production by strain B of more arginine-biosynthetic enzymes in the presence of arginine. To impose our limited logic on this predicament, let us pretend that *E. coli* did in the past use such a pathway to degrade arginine and that present-day strain B may have lost the degradative use of the pathway while retaining the "catabolite repression" type of control. In this merely speculative context, it may be suggestive to mention that apparently only the arginine pathway in *E. coli* B possesses control different from end-product repression: the other biosynthetic pathways tested so far (pyrimidines, purines, histidine, and tryptophan, to my knowledge) are all under the more common control by end-product repression.

In any case, whatever its past history, the existence of wild-type *E. coli* B offers evidence that a control system different from end-product repression may replace it and have equivalent survival value in natural selection. Actually, it was found (6) that the ability of arginine to repress enzyme synthesis is not essential for controlling arginine synthesis, but the level of the enzymes is critical. Arginine synthesis is controlled well only in strains in which the level of the en-

zymes is kept low. End-product repression, which appears to control enzyme levels in most *E. coli* strains, thus plays an important role not only in the economy of protein formation but also in the control of metabolite biosynthesis. Wild-type *E. coli* B has an alternative mechanism for limiting enzyme levels, and thus possesses an equally efficient control of arginine synthesis, whereas constitutive mutants deprived of both end-product repression and catabolite repression are overproducers of arginine.

The value of induction for the survival of an individual cell is self-evident; more subtle is the recognition of the evolutionary survival value of repression. The fact that natural selection may result in survival of strains with different types of control of the same pathway, but with a control unit nevertheless, is very good evidence of the biological significance of these units.

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